ABSTRACT

CHEMISTRY

ADAWAY, JEANETTE B.A. ALBANY STATE COLLEGE, 1974

CONFORMATIONAL STUDIES ON TURKEY LIVER FRUCTOSE 1,6-BISPHOSPHATASE USING THE FLUORESCENT HYDROPHOBIC PROBE, 1-ANILINONAPHTHALENE-8-SULFONATE.

Advisors: Professors G. S. Owen and Joe Johnson, Jr.

Thesis dated December 1976

The fluorescence emission of the hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS) was used to monitor conformational changes of turkey liver fructose 1,6-bisphosphatase (FbPase) as a function of changes in pH, addition of substrate (fructose 1,6-bisphosphate), inhibitor (adenosine monophosphate), and the divalent cations Mg^{2+}, Mn^{2+}, and Co^{2+}. Changes in the ANS fluorescence emission spectra indicate that conformational changes in the enzyme occur under the above conditions. The fluorescence emission of the hydrophobic probe was markedly increased in the presence of turkey liver FbPase. All enzyme modifications reflect conformational changes in the enzyme.

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ACKNOWLEDGEMENT

I would like to extend my most grateful appreciation to Drs. G. S. Owen and Joe Johnson, Jr. for their guidance and assistance throughout my research. Many thanks are extended to the entire faculty of the Atlanta University Chemistry Department for a learning experience that can not be forgotten. To Dr. G. Bayse a debt of gratitude is extended for the use of the fluorometer.

INTRODUCTION

A specific phosphatase, fructose 1,6-bisphosphatase (FbPase) present in the liver, kidney, and skeletal muscle of mammals and other vertebrates, catalyzes the hydrolysis of fructose 1,6-bisphosphate to yield fructose 6-phosphate (FbP) and inorganic phosphate. The enzyme was first described by Gomori (1), who separated it from other nonspecific phosphatases present in liver and kidney extracts and showed it to be inactive in the absence of Mg^{2+}. This requirement for a divalent cation can also be satisfied by Mn^{2+} (2).

FbPase from a variety of sources has been shown to be an allosteric enzyme subject to reversible inhibition by adenosine-5'monophosphate (AMP) and substrate (FbP). Evidence for the existence of four interacting sites for AMP binding and four independent sites for substrate binding per molecule of enzyme has been shown (3).

FbPase plays an important role in the regulation of gluconeogenesis. This process is the conversion of pyruvate to glucose and is the most important common pathway in the biosynthesis of monosaccharides and polysaccharides. The pathways leading from glucose-6-phosphate to other products differ widely among different organisms. The capacity to form free glucose is relatively limited; it is present in some plants and in the liver, kidney and small intestines of vertebrates.

It has also been shown that FbP markedly enhances the affinity of turkey liver FbPase for AMP (4). The formation of these intermediate binary and ternary complexes was demonstrated in the absence of added divalent cations required for substrate hydrolysis. This increased affinity was interpreted to be a direct result of conformational change in enzyme structure produced by the binding of substrate. Further evidence to support such a conformational change has now been found by the studies of the effect of substrate on the fluorescence emission of the enzyme in the presence of the hydrophobic probe, 1-anilinonaphthalene-8-sulfonate (ANS).

1-Anilinonaphthalene-8-sulfonate and certain other derivatives of aminonaphthalenesulfonic acids have very low quantum yields ($\emptyset$=0.004) in water, although they have relatively high fluorescence quantum yields when dissolved in organic solvents, e.g., in ethanol $\emptyset$=0.037, or in aqueous solutions of proteins (5). The mechanism of fluorescence of this organic dye (ANS) is described in this work. ANS fluorescence was shown to be enhanced by solvents of low dielectric constant or, to a greater extent, by solvents of low viscosity. It was proposed that the ANS fluorescence observed in protein solutions resulted from the binding of the fluorophore to hydrophobic sites upon the protein surface. This interpretation is supported by several other lines of evidence. For example, ANS is bound firmly in the hydrophobic heme crevice of myo-

globin (6). Many proteins enhance ANS fluorescence with increased efficiency after hydrophobic amino acid side chains exposed by thermal denaturation (7). These findings suggest that ANS and other structurally related compounds could be used as probes of conformational changes in proteins.

During this investigation, it became necessary to examine the ANS binding sites of turkey liver FbPase. In this work evidence is provided for the existence of sites in FbPase that have a high affinity for ANS. These sites are not a part of the enzyme active site, although the binding of substrate (FbP) to the active site of FbPase is accompanied by large changes in ANS fluorescence. It is proposed that these changes in ANS fluorescence result from conformational changes in the enzyme induced by the binding of substrate.

The binding of ANS to FbPase allows the changes in the enzyme structure to be monitored. This work will present observations on enzyme conformation upon changes in pH, and the addition of substrate (FbP) and inhibitor (AMP). Also, the effects of the divalent cations Mn^{2+}, Mg^{2+}, Co^{2+} and other enzyme modifiers on the ANS fluorescence will be reported.

LITERATURE REVIEW

Properties of Avian Fructose-1,6-Bisphosphatase

There has been very little work done with FbPase from avian species as compared with the amount of work done on mammalian FbPase. Han et al. reported the temperature of thermal inactivation of chicken liver FbPase (8). They concluded that both AMP and FbP increased the thermal stability of the enzyme, with a greater effect being observed with AMP. Marquadt and Olson also reported very similar results (9,10). They concluded that the stabilizing effect of the compounds (FbP and AMP) would induce changes in unheated avian FbPase. These compounds also stabilized FbPase following heat treatment. The latter effect is thought to be a reflection of the abilities of the compounds to prevent irreversible changes in the secondary or tertiary structures of the avian enzyme (9,10).

In our laboratory, the effect of pH on the catalytic activity of avian FbPase has been reported by several workers (4). They found that in the absence of chelating activator, purified turkey liver FbPase has optimum activity at pH 8.3-8.5 when assayed either at 30° or 42°, which is near the physiological temperature of turkey. The purified turkey liver FbPase showed a high affinity for FbP. It also required divalent cations (Mg^{2+} or Mn^{2+}) for catalytic activity and was strongly inhibited by AMP (4). It has also been demonstrated in our

laboratory that the FbPase from two different avian species (chicken and turkey) are very closely related (4,8). Enzymes from chicken and turkey have similar molecular weights, amino acid compositions, and electrophoretic mobility. Turkey liver FbPase yields a homogeneous FbPase when purified by the method of Han et al. (4). The enzyme has a molecular weight of 144,000 and may exist as four identical subunits.

Fluorescence of Bound Dyes: "Fluorescent Probes"

Changes in the fluorescence properties of dyes absorbed or covalently attached to macromolecules may signal changes in the microenvironment of the dyes due to configurational changes in the larger molecule. Dyes used in this way have been called "fluorescent probes" (11), a term that is analogous to "reporter group" (12) which is defined as a chromophore that undergoes changes in absorption characteristics as a function of environmental perturbations.

Lippert (13) has explained the spectral shift of the fluorescence band, compared with the absorption band, in terms of dipole-dipole interactions in the excited state. According to this generally accepted theory, the dipole moment of most molecules increases on excitation; this in turn requires a reorientation of the solvent dipoles forming a shell about the fluorophore. Highly polar solvents require the expenditure of relatively large amounts of energy to accomplish the reorientation, with the result that the emission

that finally appears has shifted to wavelengths of lower energy as compared with the absorption band. In less polar solvents the solvent reorientation energy is much less, and hence emission is at shorter wavelengths (higher energy).

It has long been known that covalently bound fluoresecent dyes change their characteristics in response to configurational changes. Steiner and Edelhoch (14) found that the lifetime (13.8nsec) of the fluorescence of 1-dimethylaminonaphthalen-5-sulfonic acid (DNS) attached to globulin decreased to 8nsec on denaturation with urea. They also found that the fluorescence of a fluorescein label on bovine serum albumin (BSA) diminished in the alkaline pH region where the protein unfolds (15).

The use of noncovalently bound dyes as fluorescent probes of protein structure is based on the finding that certain dyes are only weakly fluorescent when free in aqueous solution but strongly fluorescent when absorbed onto certain proteins (15-17). The dyes ANS and TNS (6-p-toluidinaphthalene-2-sulfonate) were reported to be nearly non-fluorescent in water but highly fluorescent when bound to BSA or denatured proteins (5).

The anilinonaphthalenesulfonates were studies by Forster (19), who postulated that planarity of the two ring systems was necessary for augmentation of fluorescence intensity. However, this idea was discarded when it was realized that a

change in solvent polarity alone could cause the characteristic high quantum yield and blue shift (6,18). Because the fluorescence of protein-bound ANS could be mimicked by placing the dye in nonpolar solvents, the concept arose that spectral analyses of the fluorescence could give information on the "polarity of the binding site."

Turner and Brand (20) studied the influence of solvent polarity on the fluorescence of the 1,5-, 1,7-, and 1,8- isomers of ANS and TNS. There was a strong correlation between the position of the fluorescence peak and the polarity of the solvent.

Some of the problems in interpreting the spectra of fluorescent probes seems to stem from overemphasis on polarity in applying Lippert's theory of spectral shifts (13). These shifts are determined by the energy expenditure for solvent reorientation during the lifetime of the excited state. The dielectric constant, a measure of polarity, is sometimes assumed to be the main determinant of the fluorescence characteristics; but there are times when solvent polarity and solvent-reorientation energy do not parallel each other. The effect on fluorescence of changes in viscosity rather than in the dielectric constant have been examined (21). Using mixtures of methanol and 1,2-propanediol, which both have dielectric constants of 33, Winker was able to vary the viscosity from 0.6 to 33cP. The fluorescence of p-amino-

hippurate (PAH) increased in intensity by a factor of 2.5 over this range, and there was a blue shift. More recently it was shown that a fluorescent probe, 2,6-MANS (N-methyl derivative of TNS), placed in a highly polar environment at a low temperature gave spectra similar to those obtained from nonpolar organic solvents (22). Clearly these results are due to the inhibition of solvent relaxation by high viscosity and low temperature.

Similarly, a fluorescent probe on a protein may be near very polar groups and would not be affected by them if they are immobilized and unable to interact with the excited-state probe. The original idea of Forster (19) that the planarity of the probe molecule may be important has been revived to some extent by the work of Camerman and Jensen (23,24), who determined the crystal structure of TNS by X-ray diffraction. They found that electron delocalization over the two ring systems is possible when the rings are nearly planar. Slight deformation of the structure of the probe molecule would therefore greatly perturb the fluorescence, and the authors suggested that it is not safe to conclude that these probes are sensitive only to binding-site polarity.

When absorbed onto calf-thymus histone, ANS gives a strong fluorescence (25,26). Laurence (26) finds that the fluorescence is like that of ANS bound onto BSA and therefore characteristic of nonpolar binding sites. However, no strong

binding sites were actually found. It seems possible that the ANS could be bound electrostatically to the many positively charged basic groups on histones, in an environment unconducive to solvent relaxation.

In spite of questions on the interpretation of probe data, these dyes have been useful in studying alteration in protein structure. Callaghan and Martin (16) detected the denaturation of serum albumin by the loss of its ability to form the characteristically fluorescent complex with ANS. Similarly Chen (27) used ANS to show that a defatting procedure using charcoal did not denature serum albumin; this was shown by retention of several properties of the ANS-BSA complex. Gally and Edelman (7) found that Bence-Jones protein could enhance ANS fluorescence after denaturation. Green (28) found that avidin had a hydrophobic binding site for ANS, presumably the same site that binds biotin; however, the quantum yield of ANS fluorescence was much less than in the ANS-BSA complex.

Stryer (6) presented evidence that ANS can bind to apomyoglobin and apohemoglobin in the heme crevice. Whereas it had been reported that ANS quantum efficiency was 0.004 and 0.75 in water and on BSA, respectively (29), Stryer found that ANS on apomyoglobin had a quantum yield of 0.98 (6). Hsu and Woody (30) showed that ANS and other dyes had optical activity when bound to apohemoglobin. Indeed, the binding

was so rigid in a preferred orientation that absorption and circular-dichroism spectra could be used to resolve individual vibrational transitions.

The dye ANS was found by K. Brand (31) to bind to transaldolase, with a marked enhancement of fluorescence. The substrate, FbP, reduced the fluorescence without displacing ANS from the complex. A configurational change induced by substrate was postulated.

Previous work has been done on rabbit liver FbPase with the fluorescent hydrophobic probe, ANS, by Pogell and coworkers (32,33). They presented evidence that reflects changes in enzyme conformation and found that there are three properties of FbPase which are specifically and markedly changed in the presence of low concentrations of substrate: (a) a decreased binding to carboxymethycellulose; (b) the enhanced affinity of the enzyme for its allosteric inhibitor, AMP; and (c) the decreased emission of the enzyme-ANS complex.

Fluorescence and the Phenomenological Approach

Fluorescence is an exceedingly sensitive analytical tool. Application of fluorescence assay does not require knowledge of the theoretical background of the phenomenon. However, some information concerning the physical principles of fluorescence is helpful in understanding and extending its use. This technique is very useful in detecting changes in protein conformation as a function of hydrophobic binding studies.

These studies have provided a new insight into the kinetics, structure and interactions of proteins (5,34).

An oversimplified definition of fluorescence is that it is the immediate emission of light from a molecule or atom following the absorption of radiation. Fig. 1 shows in a crude fashion how a substance can exhibit fluorescence (35). If the molecule is in the lowest vibrational level of the electronic ground state, its internuclear distance will most likely be about that shown by point (A) in the figure. This is dictated by the form of the vibrational wave function, which will give a maximum probability for this internuclear distance. If an electronic excitation occurs, it will happen so quickly that no appreciable motion of the massive and sluggish muclei will have taken place (the Frank Condon Principle). Thus, we should expect the transition to be described by a nearly vertical line (A→B) in the figure, and the most likely transitions will be those that lead to vibrational states that have maximum probability of internuclear distance close to the initial distance. In the example shown, the form of the potential energy curve for the excited state is such that the first vibrational level in this state will be favored. Two things may now happen: (1) all of the excitation energy may be lost in a nonradiative manner, or (2) the molecule may lose some energy as heat and thus reach the lowest vibrational level of the excited state and then reradiate

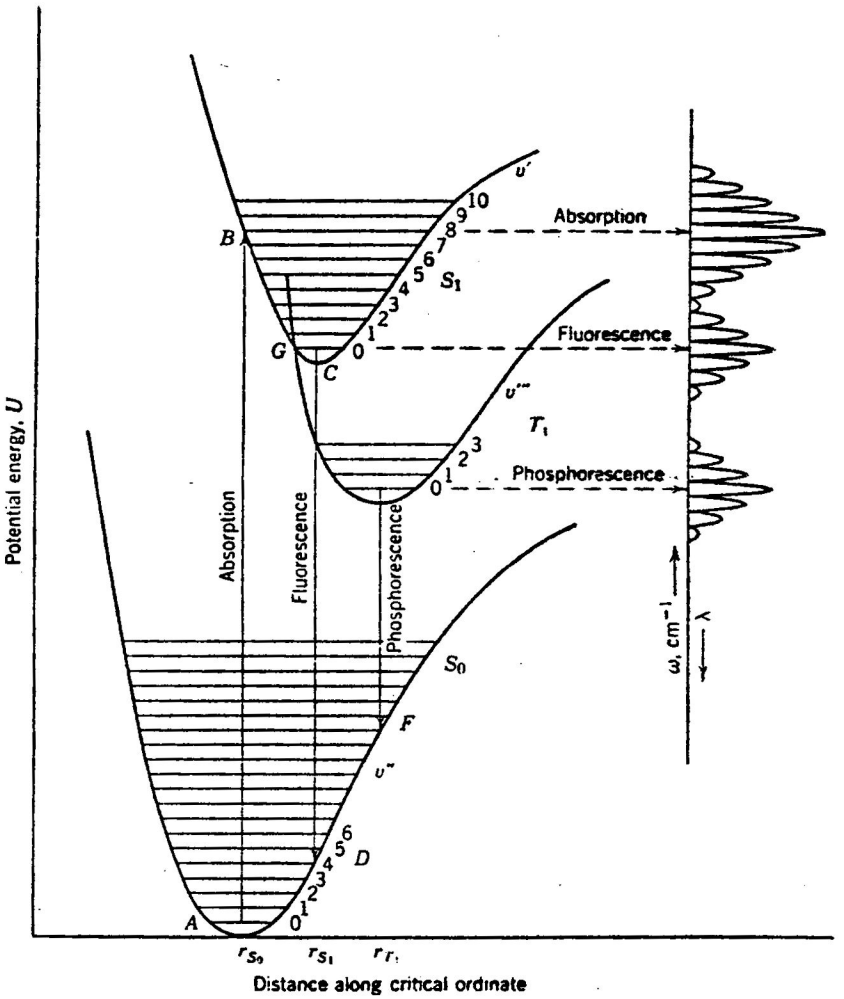


Fig. 1. "Potential Energy Diagram."* The shape of the hypersurface along a critical coordinate for the ground state S_0 and the first excited singlet S_1 and triplet T_1 states of a representative organic molecule in solution are given. G is a point of intersystem crossing, $S_1 \rightarrow T_1$.

*Taken from J. Calvert and J. Pitts, Jr., "Photochemistry," John Wiley & Sons, Inc., New York, N. Y. 1967, p. 274.

(line C→D). When this happens, the emitted light will be of lower frequency (and thus longer wavelength) than the exciting light, and fluorescence will be observed. The theory of fluorescence has been described in greater detail (34).

Energy Transfer

When applying fluorescence assay there are two phenomena which are particularly important. They are "energy transfer" and "fluorescence polarization." Only the former will be discussed here.

The transfer of electronic excitation energy from one molecule, or portion of a molecule, to another is now known to be such an efficient process that in many systems it may occur at every encounter. Furthermore, it is known to occur widely in nature even over distances as great as 50A° or more. Largely through the efforts of Forster (36) it is now recognized that transfers can proceed by a radiationless mechanism whereby the molecules which are originally excited (primary oscillators) transmit the excitation energy to recipient molecules (oscillators) at a distance. The interaction is due to dipole-dipole coupling and not to the overlap of electron orbitals (37).

Quantum Yield

The polarity of the binding region can be evaluated in terms of two parameters, the emission maximum and the quantum yield of fluorescence of bound dye.

The percentage of the absorbed energy which is re-emitted as fluorescence is indicated by the term "quantum yield of fluorescence," designated as $\emptyset$, where

$$\emptyset = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}}.$$

In the absence of any perturbations, the emitted fluorescence is equal to the absorbed radiation and $\emptyset=1$. The lifetime of the excited state under these ideal conditions is referred to as τ_o and can be estimated from absorption data (38). The actual value of $\emptyset$ under practical conditions can be derived from the equation

$$\emptyset = \tau/\tau_o$$

where τ has been experimentally determined.

EXPERIMENTAL

Materials

D-fructose 1,6 bisphosphate (FbP) and adenosine-5'monophosphate (AMP) were obtained from Sigma Biochemicals, St. Louis, Mo. The ammonium salt of 1-anilinonaphthalene-8-sulfonic acid (ANS) obtained from the Sigma Chemical Co., St. Louis, Mo., was twice recrystallized from hot water according to the method of Weber and Young (29). The crystals were dried at 50° for several hrs and stored in the dark. Concentrations of this reagent in aqueous solutions were determined assuming an extinction coefficient of $4.95X10^3 cm^2/m$-mole at 350nm (29). Tris-HCl, cobalt sulfate, magnesium sulfate and manganese chloride were purchased from the Fisher Scientific Co. All other chemicals used were of reagent grade.

Homogeneous turkey liver FbPase was obtained from P. Han and was prepared by his method (4). Protein concentration of the purified enzyme was determined spectrophotometrically at 280nm. A solution containing 1.0mg (dry wt) of purified turkey liver FbPase per ml in a light path of 1.0cm has an absorbance of 0.75, and this was used as a standard.

All reagents for fluorescence experiments were dissolved in Chelex treated distilled water. Chelex treatment was used to remove trace amounts of heavy metals (e.g., zinc).

Methods

FbPase activity at pH 7.5 was determined spectrophotometrically as previously described (4).

Fluorescence emission measurements were carried out at room temperature (25°) in a Perkin-Elmer Model 204 fluorescence spectrophotometer, a grating instrument designed to automatically record fluorescence emission intensity vs. wavelength in the ultraviolet and visible regions for a variety of compounds. A fixed slit of 10mμ width was used. Automatic recording of intensity vs. wavelength was performed on a Perkin-Elmer Model 056 strip chart recorder. The sample compartment holds 4 10mm square cells in a rotable turret selected by an external lever.

Samples in a usual vol of 3ml containing 50mM Tris-HCl buffer, pH 7.5, were placed in a 10mm square fused quartz cell, and properly aligned into the cell compartment. The excitation and emission intensities are expressed in arbitrary fluorescence units after corrections for the appropriate ANS blank.

Equilibrium dialysis was carried out in 1ml dialysis cells (Chemical Rubber Company, No 547695) with continuous mixing in the dark at room temperature. Concentrations of ANS in samples from both sides of the dialysis membrane were determined fluorometrically after a 1:10 dilution in ethanol.

Absorption Measurements.--Spectrophotometric absorption measurements were obtained using a Coleman Model 124 double beam grating U.V.-visible spectrophotometer and a Cary 17 recording spectrophotometer.

Fluorescence Studies.--The fluorescence emission spectra of the purified homogeneous FbPase-ANS complex was obtained under the following conditions: (a) FbPase-ANS complex plus 10mM AMP and 10mM FbP; (b) the FbPase-ANS complex plus the additions of 0.5mM, 1mM, 5mM, and 10mM solutions of FbP followed by identical amounts of AMP at pH 7.5; (c) the FbPase-ANS complex plus 10mM solution of AMP followed by the addition of a 10mM solution of FbP; (d) the FbPase-ANS complex at pH's 6.0, 7.0, 8.0, and 9.0; (e) the FbPase-ANS complex plus 10mM solutions of Cobalt, Manganese, and Magnesium; (f) the FbPase-ANS complex plus substrate (10mM FbP) along with the metal co-factor, (Mn^{2+}); and (g) the FbPase-ANS complex plus urea additions, ranging from 2M to 10M in increments of 2M. The instrument used was as reported in "Materials" above.

RESULTS AND DISCUSSIONS

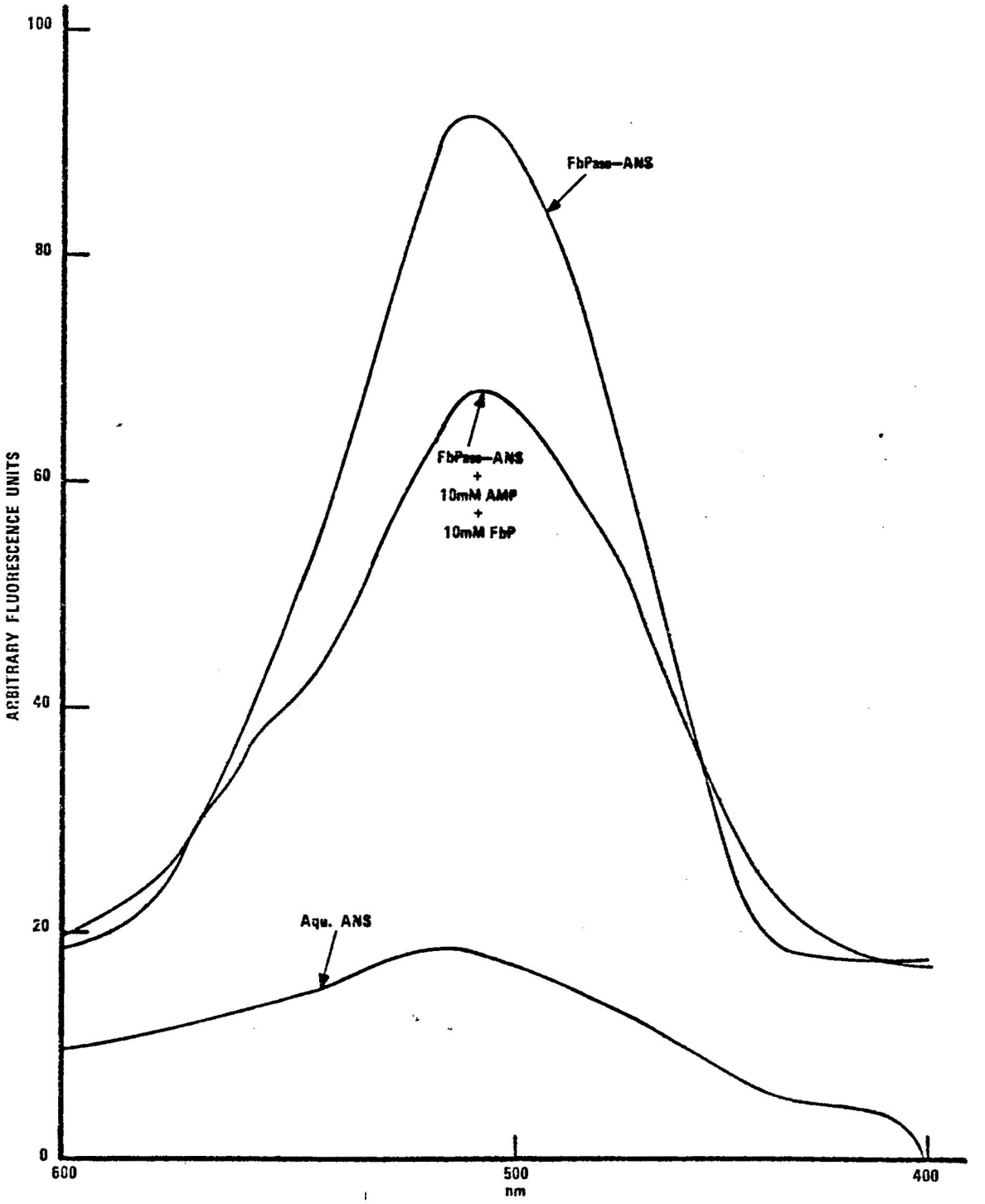
Fluorescence Properties of FbPase-ANS Complex

The addition of FbPase to a dilute solution of ANS in Tris-HCl buffer at pH 7.5 resulted in a large increase in fluorescence emission (Fig. 2). The excitation maximum was at 378-380nm and the emission peak appeared around 508nm. ANS or FbPase alone gave negligible emission under these conditions. The fluorescence intensity of the above system (ANS-FbPase) was markedly quenched by high concentrations of FbP and low concentrations of AMP with no significant change in the spectral position, suggesting that these effector molecules produced a pronounced alteration in the enzyme structure. Neither compound had any absorption at either the excitation or the emission region nor did they have any effect on the fluorescence emission of ANS in the absence of enzyme.

The Effect of Varying Concentrations of AMP and FbP on the ANS-FbPase Complex

The effect of varying the concentration of substrate (FbP) and the allosteric inhibitor (AMP) on the fluorescence emission of the FbPase-ANS system are shown in Fig. 3-6. The magnitude of the decrease with high concentrations of FbP was much less than that with AMP. Thus, the lower the concentration of AMP the more the fluorescence intensity of the enzyme-complex increased. A reversible substrate induced

Fig. 2. Fluorescence Emission Spectra of FbPase-ANS with Inhibitor (AMP) and Substrate (FbP) at pH 7.5.


100
80
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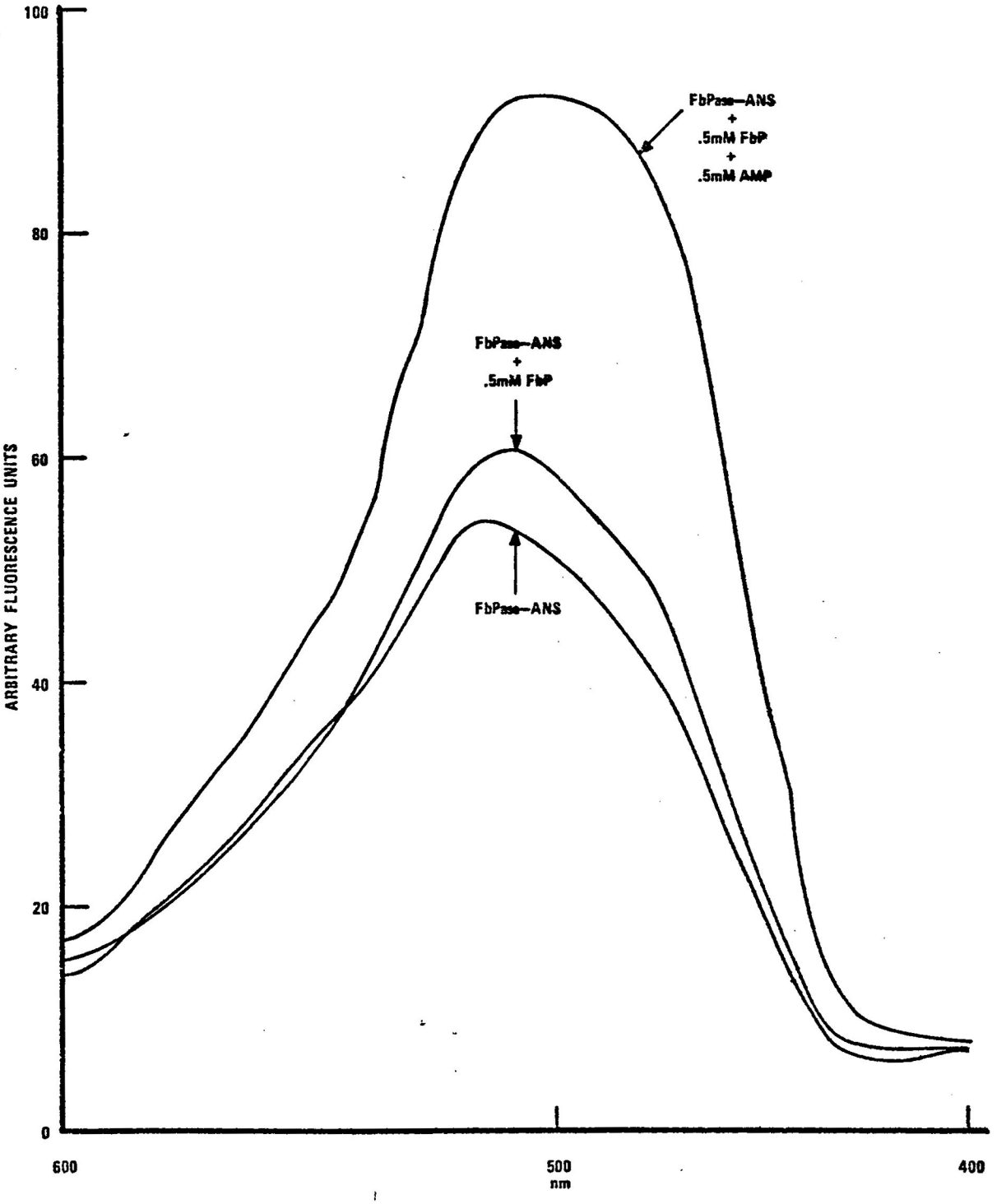
ARBITRARY FLUORESCENCE UNITS
FbPase–ANS
FbPase–ANS
+
10mM AMP
+
10mM FbP
Aqu. ANS
600
500
400
nm

Fig. 3. Fluorescence Emission Spectra of FbPase-ANS with Inhibitor (0.5mM AMP) and Substrate (0.5mM FbP).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.5mM FbP
+
.5mM AMP
FbPase–ANS
+
.5mM FbP
FbPase–ANS
600
500
nm
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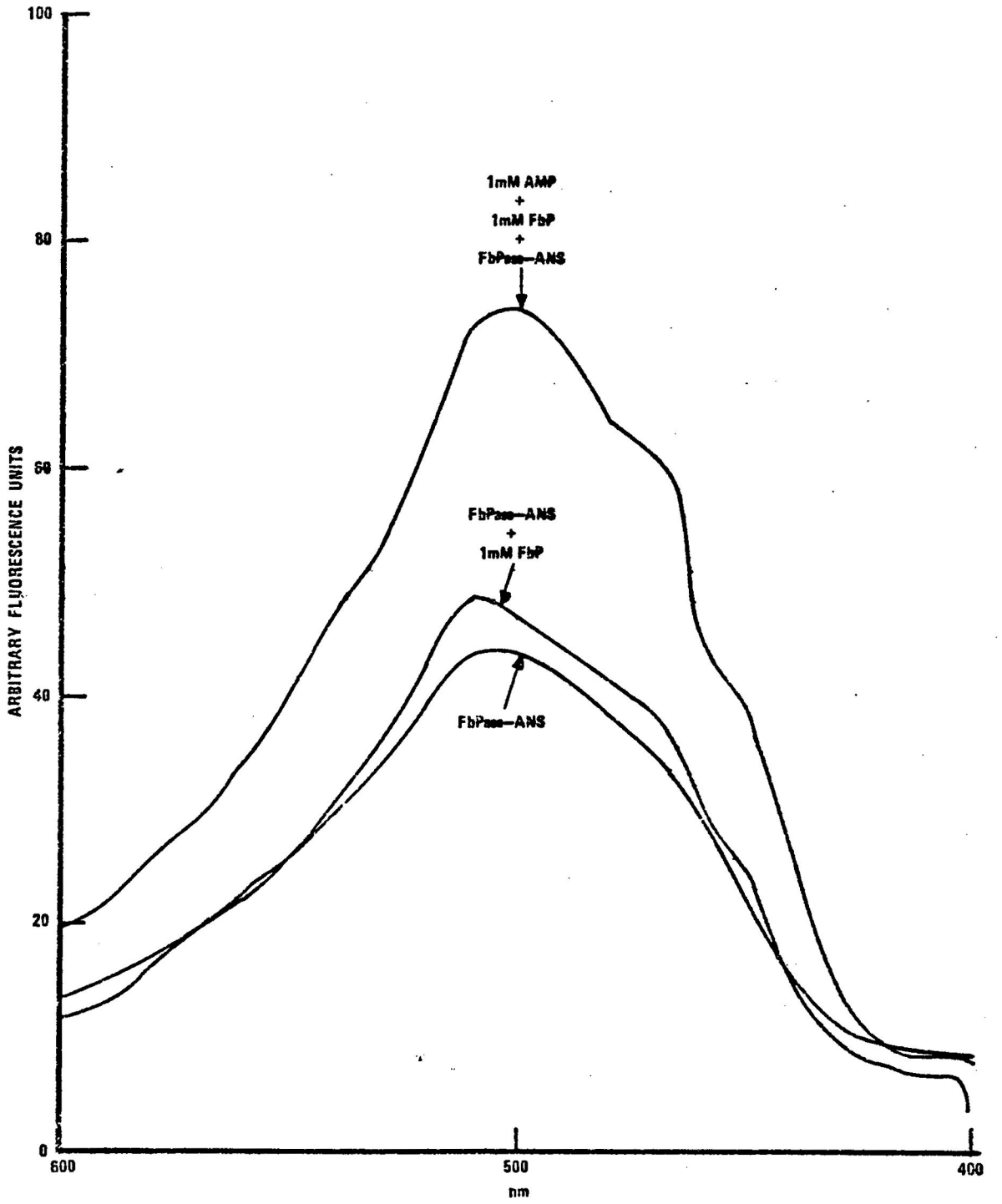
Fig. 4. Fluorescence Emission Spectra of FbPase-ANS with Substrate (1mM FbP) and Inhibitor (1mM AMP).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1mM FbP
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FbPase–ANS
FbPase–ANS
+
1mM FbP
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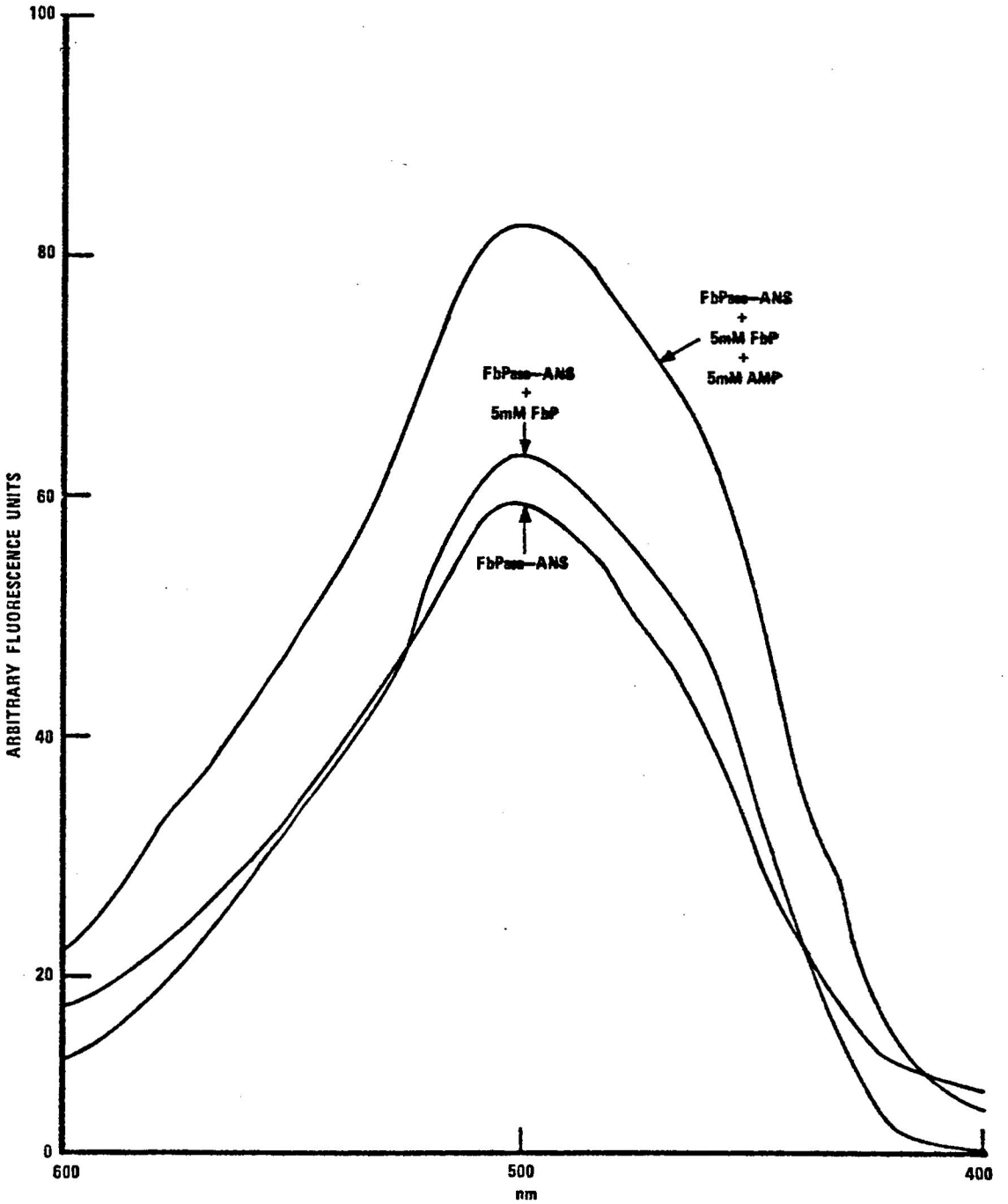
Fig. 5. Fluorescence Emission Spectra of FbPase-ANS with Substrate (5mM FbP) and Inhibitor (5mM AMP).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5mM FbP
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5mM AMP
FbPase–ANS
+
5mM FbP
FbPase–ANS
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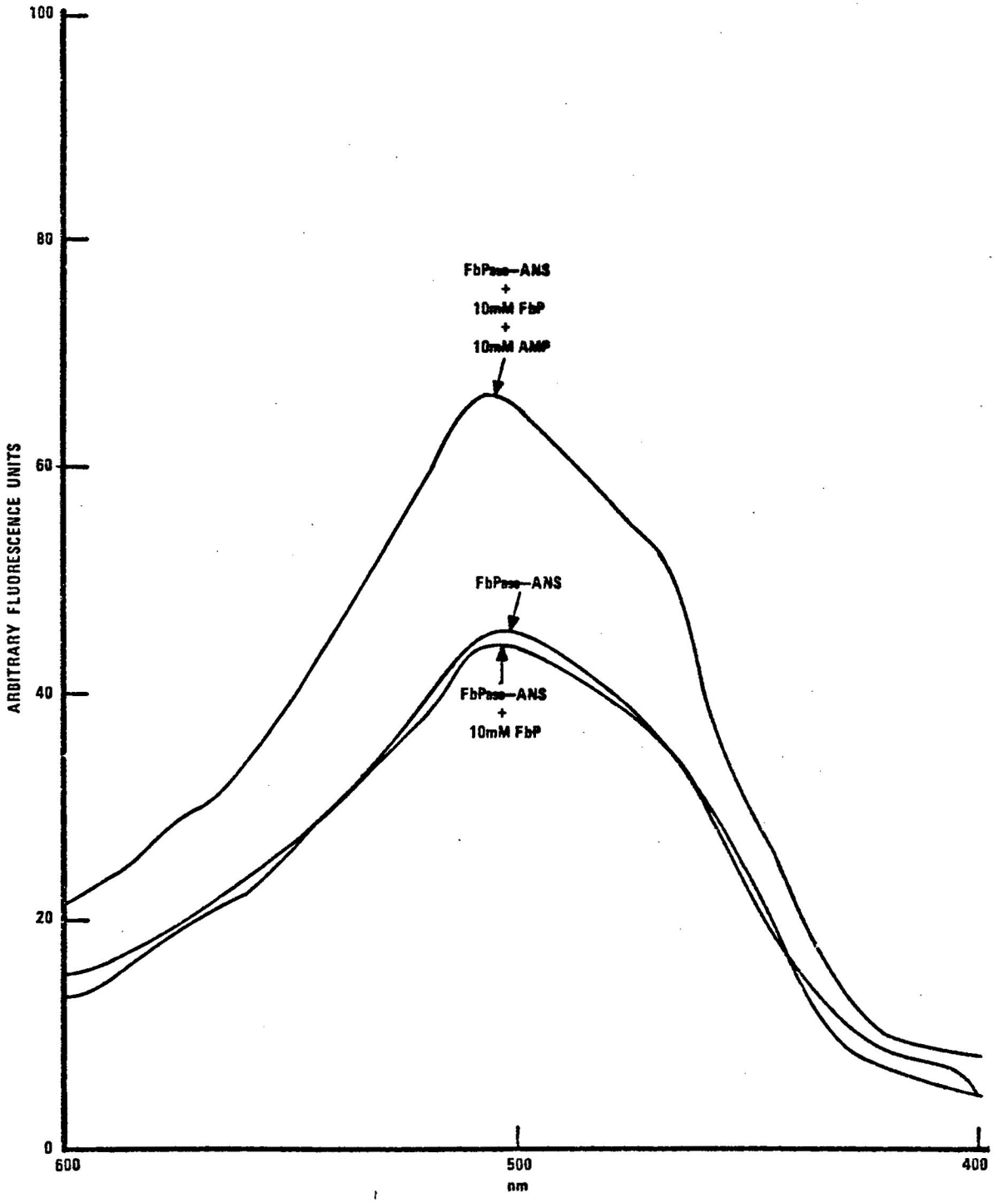
Fig. 6. Fluorescence Emission Spectra of FbPase-ANS with Substrate (10mM FbP) and Inhibitor (10mM AMP).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FbPase–ANS
+
10mM FbP
+
10mM AMP
FbPase–ANS
FbPase–ANS
+
10mM FbP
600
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nm

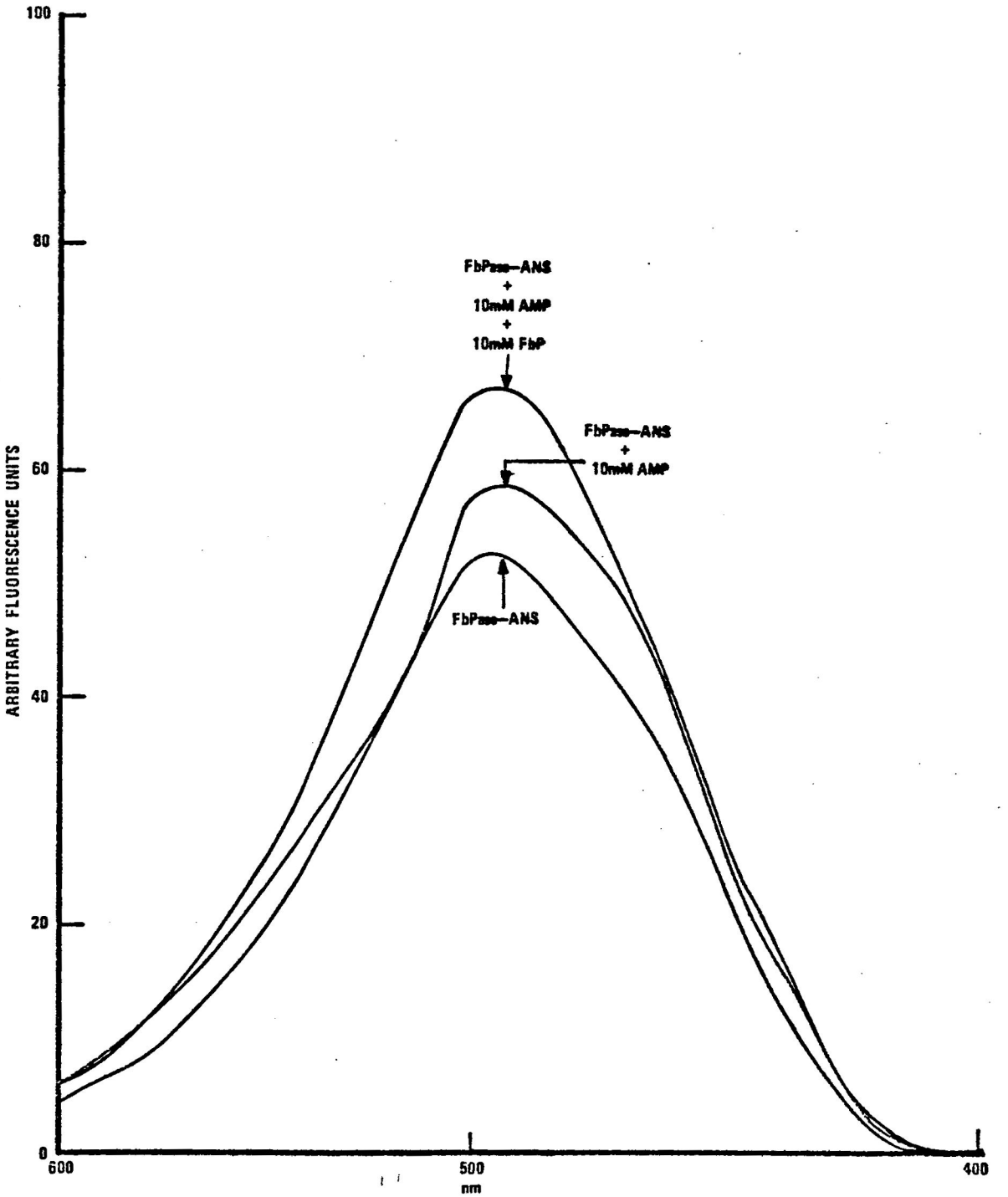
conformational change occurs upon the addition of 10mM FbP, whereas a 10mM solution of AMP caused the fluorescence intensity of the FbPase-ANS complex to decrease as compared to the lower concentrations of AMP. Thus, it appeared that substrate alone could induce large changes in enzyme configuration at high concentrations.

The addition of a 10mM solution of AMP to the FbPase-ANS complex caused a slight shift in the emission maximum to a shorter wavelength and an increase in the fluorescence emission intensity. These observations are shown in Fig. 7. When a 10mM solution of FbP was added to the system above, the emission maximum shifted back to its original wavelength, 508nm. These findings suggest that the substrate produced a pronounced alteration in the enzyme conformation. These results are quite different from those found in Fig. 6, where the addition of FbP was added before the inhibitor, AMP. Thus, it appears that the binding of substrate molecules changes the conformation of the enzyme resulting in an increased affinity for AMP and a decreased hydrophobic environment for the binding of ANS.

Effect of Urea

Fig. 8 shows the effect of varying concentrations of urea. The addition of urea to the FbPase-ANS complex caused a fluorescence quenching of the enzyme-dye complex. There is

Fig. 7. Fluorescence Emission Spectra of FbPase-ANS with Inhibitor (10mM AMP) and Substrate (10mM FbP).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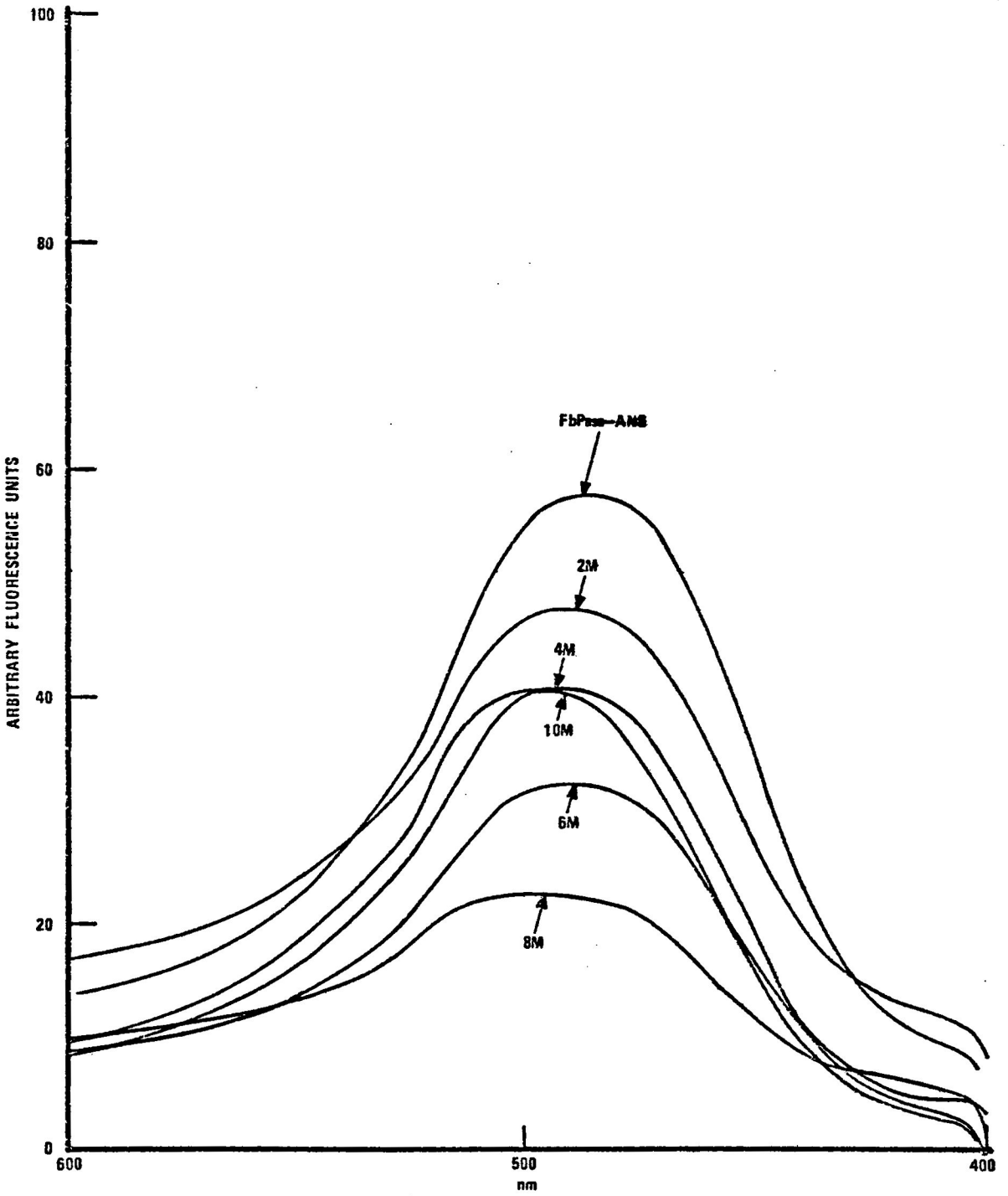
+
10mM AMP
+
10mM FbP
FbPase–ANS
+
10mM AMP
FbPase–ANS
600
500
400
nm

Fig. 8. Fluorescence Emission Spectra of FbPase-ANS with 2M, 4M, 8M, and 10M Solutions of Urea.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ARBITRARY FLUORESCENCE UNITS
FbPase-ANS
2M
4M
10M
6M
8M
600
500
400
nm

a constant decrease in the fluorescence emission of the enzyme-system as the concentration of urea increased, with the exception of the 10M sample. This concentration caused a shift to longer wavelengths, lower energy. It is only at this concentration that the enzyme seems to be denaturing. This indicates that the macromolecule has more exposed non-polar amino acids available for the ANS probe to bind.

Heat Denaturation

Denaturation of the FbPase by boiling produced a 3 to 4 fold higher fluorescence intensity, and markedly decreased the effects of subsequent addition of substrate. These results were expected because of the exposure of more hydrophobic sites upon denaturation (7).

Effect of Changes in pH

In Tris-HCl buffer, the fluorescence emission intensity of the FbPase-ANS complex increased with an increase in pH. These results are shown in Fig. 9. Such results were expected, since more hydrophobic residues are exposed upon increases in pH. It is believed that the quenched fluorescence emission in the presence of substrate will be constant over a pH range of 7.3-8.5. There were no observable shifts in emission maximum.

Reversal of Substrate-Induced Fluorescence Decrease

Fig. 10 shows the changes that occurred upon the addition of substrate (FbP) to the FbPase-ANS complex, and the

Fig. 9. Fluorescence Emission Spectra of FbPase-ANS at pH's 6.0, 7.0, 8.0, and 9.0.

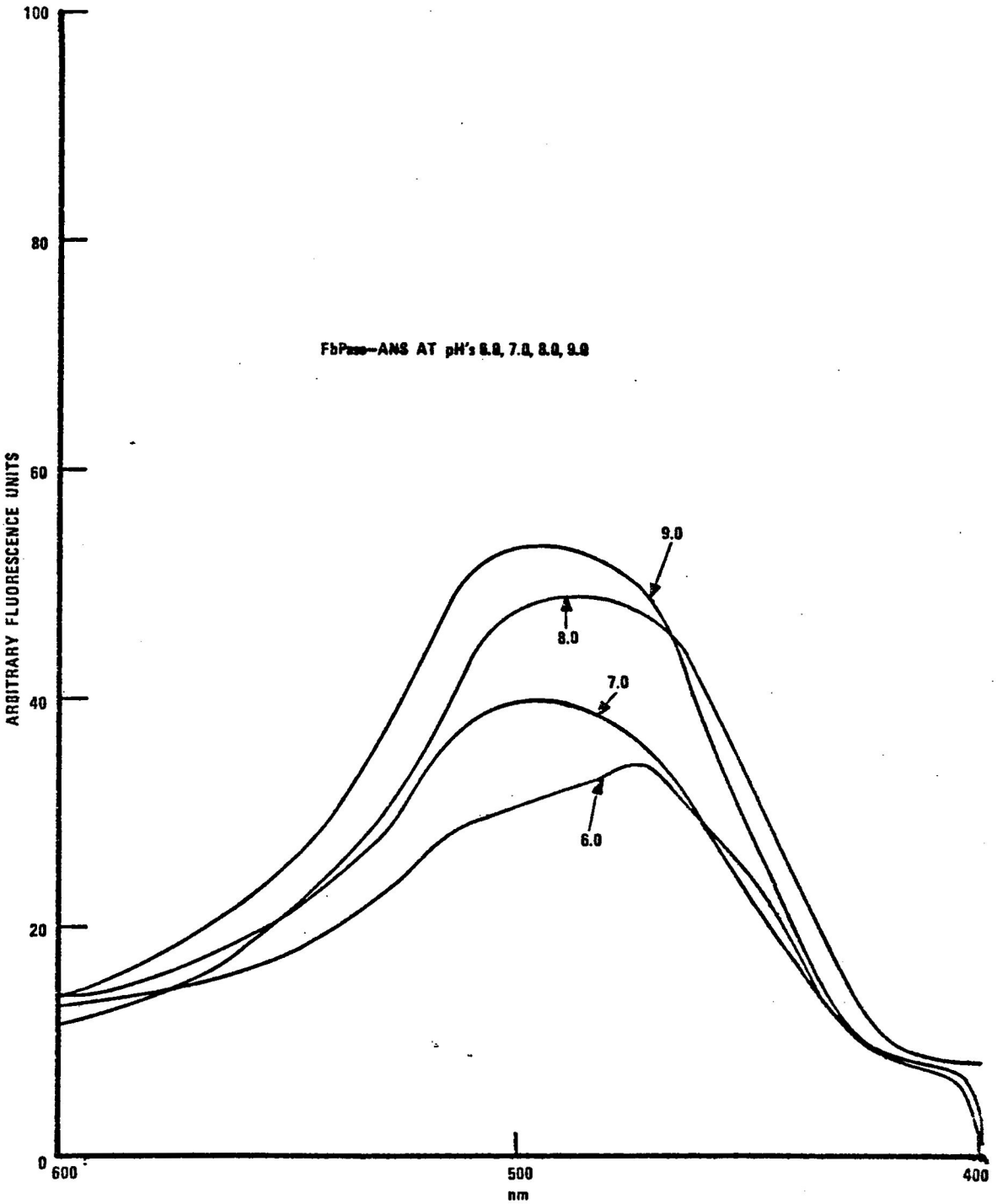

FbPase–ANS AT pH's 6.0, 7.0, 8.0, 9.0
ARBITRARY FLUORESCENCE UNITS
100
80
60
40
20
0
9.0
8.0
7.0
6.0
600
500
400
nm

Fig. 10. Reversal of Substrate-Induced Decrease in the Fluorescence Emission of FbPase-ANS at pH 7.5.

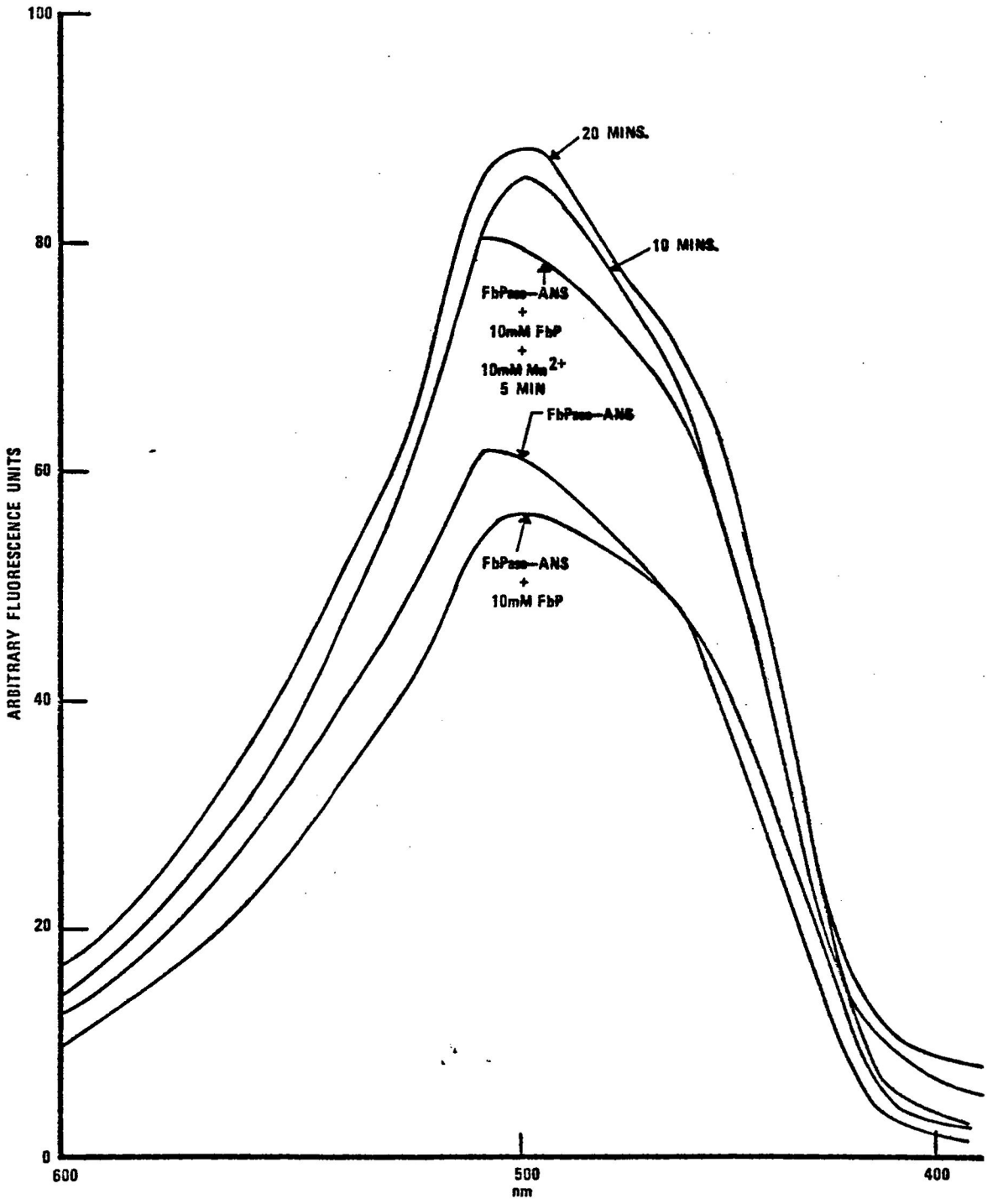

100
80
60
40
20
0
ARBITRARY FLUORESCENCE UNITS
20 MINS.
10 MINS.
FbPase–ANS
+
10mM FbP
+
10mM Mn2+
5 MIN
FbPase–ANS
FbPase–ANS
+
10mM FbP
600
500
nm
400

table below illustrates these changes in a more detailed manner. Kinetic measurements at pH 7.5 showed that high concentrations of ANS had no effect on enzyme activity, so it was possible to demonstrate a reversal of substrate-induced fluorescence quenching. Manganese ions catalyze the overall reaction at high concentrations; whereas this cation alone has very little effect on the FbPase-ANS fluorescence as may be seen in the table below. The recovery of fluorescence emission after the addition of Mn^{2+} was very rapid and almost complete in 20 minutes. The emission maximum shifted to higher energy (shorter wavelength) slightly as the recovery of fluorescence emission after the addition of Mn^{2+} was complete. This experiment again indicates that the binding of substrate induces a conformational change.

Reversal of Substrate-Induced Decrease in Fluorescence Emission.

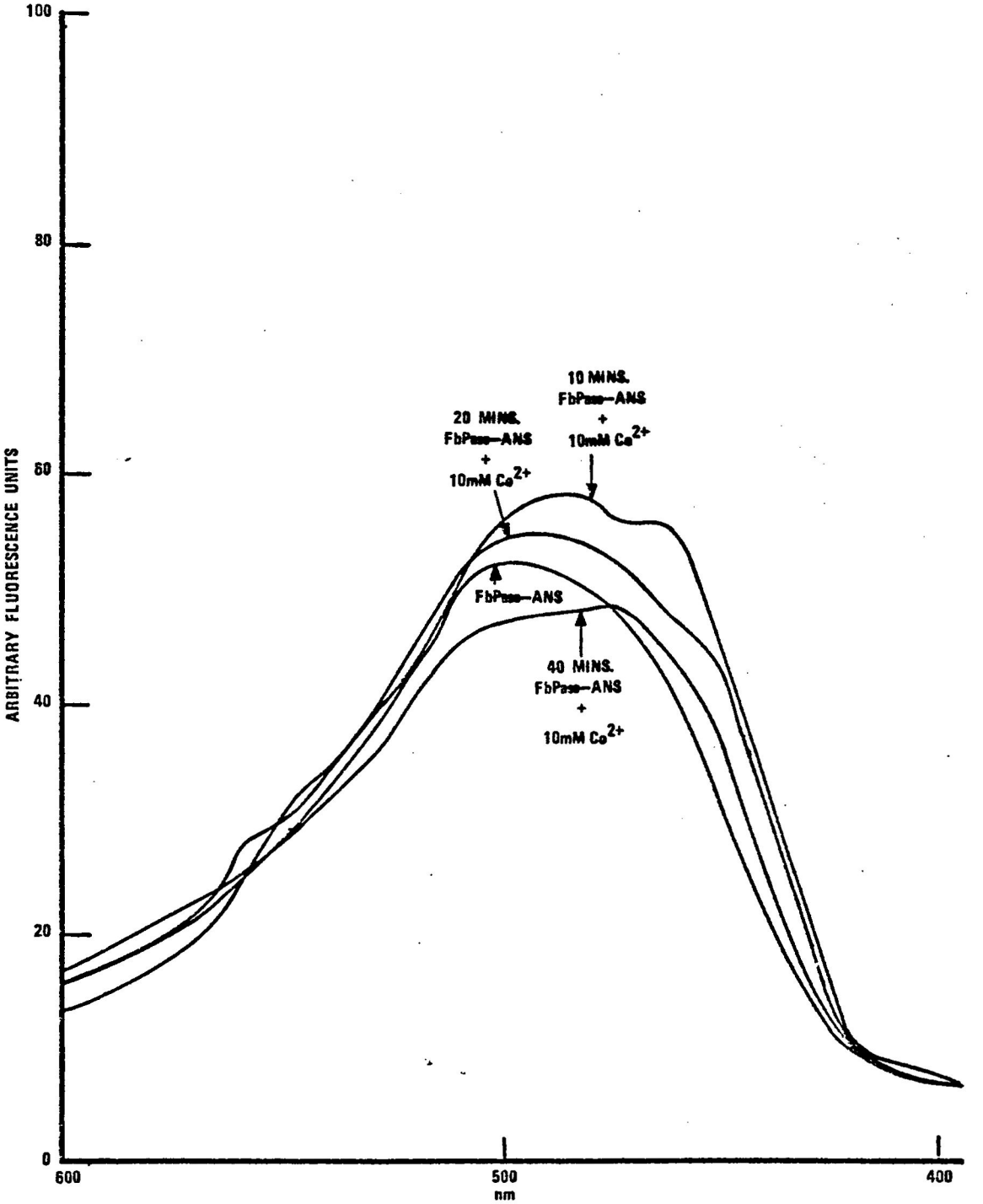
Addition		Arbitrary Units (%)
Enzyme (0.4mg/ml) + ANS (10^{-6}M)		55
Enzyme + ANS + Mn^{++} (10mM)		52
Enzyme + ANS + FbP (10mM)		49
Mn^{++} (10mM)	5 min	78
	10 min	84
	20 min	86

The Effect of the Divalent Cations (Co^{2+}, Mn^{2+}, and Mg^{2+})

The effect of the divalent cations Mg^{2+} and Co^{2+} on the fluorescence emission of FbPase-ANS was also studied. The cation, Co^{2+} quenched the fluorescence emission of the enzyme complex. The addition of Co^{2+} caused a decreased in the fluorescence emission intensity of the enzyme system with time, and shifts in the emission maximum to shorter wavelength were observed. Fig. 11 shows these results.

The cation, Mg^{2+}, caused an effect slightly different from that caused by the divalent cation, Co^{2+}, and very much similar to that caused by Mn^{2+} on the FbPase-ANS complex. The fluorescence emission decreased with time. It was noticed that when the substrate was added to the FbPase-ANS complex, the fluorescence emission increased with time in the presence of the metal co-factor, Mn^{2+}. The most plausible explanation for these findings is a conformational change induced by the binding of the substrate.

Fig. 11. Fluorescence Emission Spectra of FbPase-ANS with the Divalent Cation Co^{+2}.


100
80
60
40
20
0
ARBITRARY FLUORESCENCE UNITS
600
500
400
nm
10 MINS.
FbPase–ANS
+
10mM Ca2+
20 MINS.
FbPase–ANS
+
10mM Ca2+
FbPase–ANS
40 MINS.
FbPase–ANS
+
10mM Ca2+

CONCLUSION

The data presented, and all other spectral observations indicate that the enzyme undergoes conformational changes due to environmental perturbations. The fluorescence emission of the hydrophobic probe, ANS, was markedly increased in the presence of turkey liver FbPase.

The substrate (FbP) produced a specific and reversible decrease in the fluorescence yield. The binding of the substrate to the enzyme-ANS complex enhances the affinity of the enzyme for its allosteric inhibitor, AMP.

From these studies performed on FbPase using the fluorescent hydrophobic probe, ANS, it is apparent that there are other hydrophobic regions on the enzyme molecule that are not a part of the enzyme active or catalytic site. In relation to pH, binding of substrate and inhibitor, and all other enzyme modifiers, all evidence points to change in enzyme conformation.

REFERENCES

1. G. Gomori, J. Biol. Chem., 148, 139 (1943).

2. S. Pontremoli and B. L. Horecker, "The Enzymes" (P. D. Boyer, Ed.) Vol. IV, Academic Press, New York, N. Y., 1971, pp. 611-646.

3. S. Pontremoli, F. Grazi and A. Accorsi, Biochemistry, 7, 1655 (1968).

4. P. F. Han, G. S. Owen and J. Johnson, Jr., Arch. Biochem. Biophys., 168, 171 (1975)

5. G. Weber and D. J. R. Laurence, J. Biochem., 51, xxi (1954).

6. L. Stryer, J. Mol. Biol., 13, 482 (1965).

7. J. A. Gally and G. M. Edelman, Biochem. Biophys. Acta., 94, 175 (1965).

8. P. F. Han, V. V. Murphy and J. Johnson, Jr., Arch. Biochem. Biophys., 173, 293 (1976).

9. R. R. Marquadt and J. P. Olson, Can. J. Biochem., 52, 670 (1974).

10. J. P. Olson and R. R. Marquadt, Biochem. Biophys. Acta., 268, 453 (1972).

11. G. M. Edelman and W. O. McClure, Acc. Chem. Res., 1, 65 (1968).

12. M. Burr and D. E. Koshland, Jr., Proc. Natl. Acad. Sci. U. S., 52, 1017 (1964).

13. E. Lippert, Z. Electrochem., 61, 962 (1957).

14. R. F. Steiner and H. Edelhoch, J. Amer. Chem. Soc., 83, 1435 (1961).

15. R. F. Steiner and H. Edelhoch, Chem. Rev., 62, 457 (1962).

16. P. Callaghan and N. H. Martin, Biochem. J., 83, 144 (1962).

17. B. Alexander and G. M. Edelman, Federation Proc., 24, 413 (1965).

18. W. O. McClure and G. M. Edelman, Biochemistry, 5, 1908 (1965).

19. T. Forster, Naturwissenschaften, 33, 220 (1946).

20. D. C. Turner and L. Brand, Biochemistry, 7, 3381 (1968).

21. M. H. Winker, Biophys. J., 7, 719 (1967).

22. C. J. Seliskar and L. Brand, Science, 171, 799 (1971).

23. A. Camerman and L. H. Jensen, ibid., 165, 493 (1969).

24. A. Camerman and L. H. Jensen, J. Amer. Chem. Soc., 92, 4200 (1970).

25. G. R. Shepherd and B. J. Noland, Anal. Biochem., 11, 443 (1965).

26. D. J. R. Laurence, Biochem. J., 99, 419 (1966).

27. R. F. Chen, J. Biol. Chem., 242, 173 (1967).

28. N. M. Green, Biochem. J., 94, 23C (1965).

29. G. Weber and L. B. Young, J. Biol. Chem., 239, 1415 (1964).

30. M. C. Hsu and A. W. Woody, Biopolymers, 9, 1421 (1971).

31. K. Brand, FEBS Letters, 7, 235 (1970).

32. A. Hajime, M. G. Sarngadharan and B. M. Pogell, J. Biol. Chem., 23, 6383 (1970).

33. B. M. Pogell, Biochem. Biophys. Res. Commun., 7, 225 (1962).

34. K. E. Van Holde, "Physical Biochemistry," Prentice Hall, Inc., Englewood Cliffs, N. J., 1971, pp. 170-172.

35. J. G. Calvert and J. N. Pitts, Jr., "Photochemistry," John Wiley & Sons, Inc., New York, N. Y., 1967, p. 274.

36. T. Forster, "Fluoreszenz Organisher Verbindugen," Vandenhoeck and Ruprecht, Gottingen, 1951.

37. Sidney Udenfriend, "Fluorescence Assay in Biology and Medicine," Academic Press, Inc., New York, N. Y., 1962, p. 192.

38. Ibid., p. 17.